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PRINCIPAL INVESTIGATOR: Geoffrey J. Clark, Ph.D.

CONTRACTING ORGANIZATION: University of Louisville
Louisville, KY 40292

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Geoffrey J. Clark, Ph.D.

E-Mail: gjclar01@louisville.edu

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14. ABSTRACT

The RASSF1A tumor suppressor is frequently inactivated by aberrant promoter methylation leading to transcriptional silencing in ovarian cancer. Our preliminary data implicated loss of expression of RASSF1A in the acquisition of resistance to Taxol in ovarian cancer. We have now shown that experimental inactivation of RASSF1A does indeed induce resistance to Taxol mediated cell death, apoptosis and induction of tubulin polymerization. Furthermore, we have been able to reverse this effect by the use of a novel combination of drugs that inhibit DNA methylation to obtain a synergistic reactivation of RASSF1A expression. This provides proof of principal for the use of epigenetic therapy to overcome Taxol resistance in ovarian cancer.

15. SUBJECT TERMS
RASSF1A, Taxol resistance, Ovarian cancer

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EVALUATION OF EPIGENETIC THERAPY TO ENHANCE THE EFFECTIVENESS OF TAXOL

Introduction

RASSF1A is a poorly understood tumor suppressor that can modulate tubulin dynamics and apoptosis. It is subjected to epigenetic inactivation in approximately 50% of ovarian tumors by a process of aberrant promoter methylation leading to transcriptional silencing (1-3). Over-expression of RASSF1A promotes hyper-stabilization of microtubules reminiscent of Taxol (4;5), and previous investigations have implicated RASSF1A as playing a role in the response of cells to microtubule disrupting drugs such as nocodazole (5). This implies that RASSF1A may be necessary for Taxol to exert its full effects and might implicate loss of RASSF1A expression in the development of Taxol resistance in ovarian cancer. In an attempt to address the issue of RASSF1A expression and Taxol resistance, we performed a micro array analysis of a series of primary ovarian tumor samples that were characterized for resistance or sensitivity to Taxol. The analysis failed to identify any particular variations in RASSF1A expression (data not shown). However, the natural levels of RASSF1A expression were quite modest, and so we felt that these results might be inaccurate due to the relative insensitivity of the micro array mass screen. Consequently, we repeated the assay using qRT-PCR. This is a much more sensitive assay and produced results that showed a very strong correlation between the reduced expression of RASSF1A and Taxol resistance in primary ovarian cancer (Figure 1).

This project was designed to test two hypotheses: Firstly, that the levels of RASSF1A protein may play an important role in the response/resistance of ovarian tumor cells to Taxol. Secondly, that the use of novel combinations of drugs that promote DNA demethylation may act to enhance the effectiveness of Taxol by restoring the expression of epigenetically inactivated tumor suppressors such as RASSF1A. This would provide proof of principal for the use of epigenetic therapy in combination with Taxol to overcome Taxol resistance.

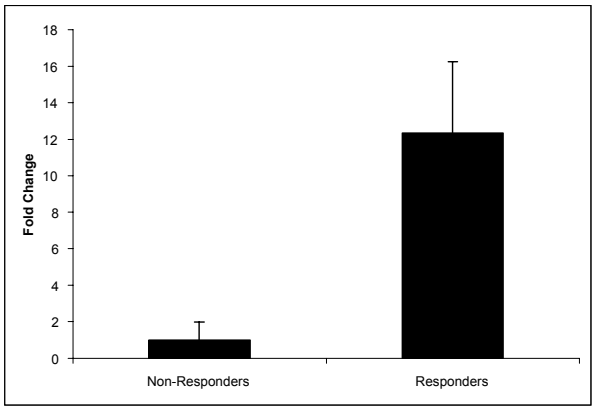


Figure 1. RASSF1A down-regulation correlates with acquisition of Taxol resistance in primary ovarian tumors:
qRT-PCR analysis of primary ovarian tumors correlates loss of RASSF1A expression with the development of Taxol resistance. Left column is relative expression of RASSF1A in Taxol resistant patients; right column is relative expression in taxol sensitive patients.

BODY

TASK1:

Establishment of matched pairs of ovarian tumor cell lines that are +/- for RASSF1A expression, followed by the analysis of their response to Taxol or Cisplatin.

A series of ovarian tumor cell lines that retain RASSF1A expression were transfected with an shRNA against RASSF1A or a vector control and stably selected. The cells were then Western blotted with our RASSF1A antibody to confirm the down regulation of the RASSF1A (Figure 2).

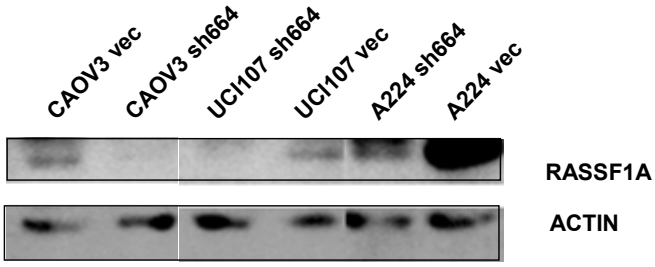


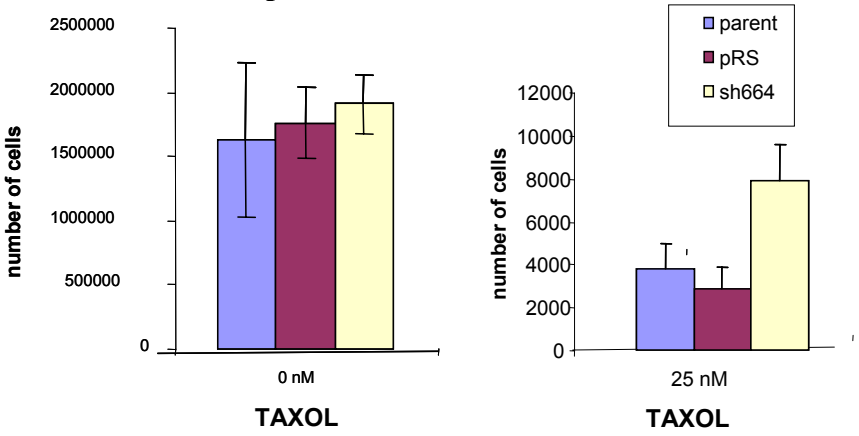
Figure 2. Knockdown of RASSF1A. Ovarian Tumor cell lines stably transfected with an shRNA (sh664) to RASSF1A or vector control. The cells were subjected to Western analysis to confirm knockdown of RASSF1A. Actin served as a loading control (lower panel).

UCI-107 (6) and CAOV3 (7) matched pairs were then assayed for their relative response to Taxol and Cisplatin. The A224 matched pair was unfortunately lost during a power loss caused by hurricane Ike.

The UCI-107 matched pair demonstrated that loss of RASSF1A had a marked effect on the response to Taxol (Figure 3). The CaOV3 cells were so sensitive to Taxol that too few cells survived the experiments to allow interpretation of the data.

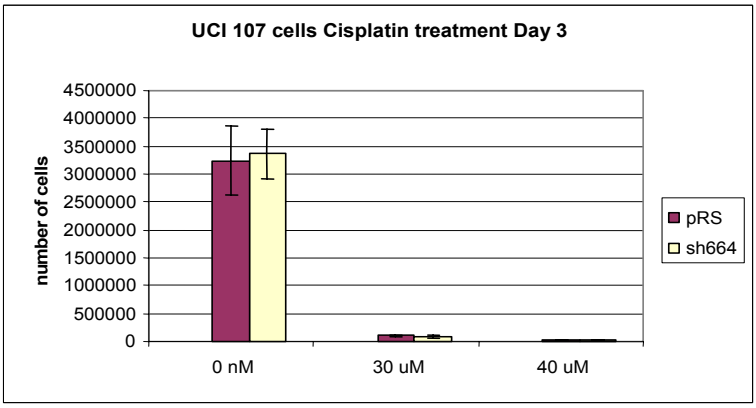
Figure 3. Effects of RASSF1A expression on the death response to Taxol.

UCI-107 cells +/- for RASSF1A were grown to 50% confluency and then treated with carrier or 25nM Taxol for three days. Cell survival was determined by counting cells in a haemocytometer. On average, approximately twice as many RASSF1A knockdown cells survived the treatment as cells transfected with the control vector. P value was .01.



The UCI-107 +/- cells were then challenged with Cisplatin. No difference in the response to Cisplatin could be detected (Figure 4). As we were unable to collect interpretable data in the Taxol assays from the CAOV3 cells, we did not incorporate this cell system in the Cisplatin assays.

Figure 4. Response to Cisplatin
UCI-107 RASSF1A +/- cell lines were challenged with Cisplatin and scored for survival after 3 days. The cells were very sensitive to Cisplatin and no differences between the RASSF1A +/- cell could be detected.



Summary:
It appears that loss of RASSF1A induces a resistance to Taxol in some Ovarian tumor cell lines. No differences in the response to Cisplatin were detected.

During this task we were surprised to find that several Ovarian tumor cell lines appeared to be naturally highly resistant to Puromycin. This made it impossible to generate stable knockdown cells with the shRNA constructs in these cell lines as the vector selection marker is puromycin. We also found that several cell lines were so sensitive to Blasticidin that we could not select stable transfectants. As some cell lines were lost during power emergencies and CaOV3 cells were so sensitive to Taxol that almost all the cells always died regardless of the RASSF1A status, we focused subsequent studies on the UCI-107 cells where we could detect a differential.

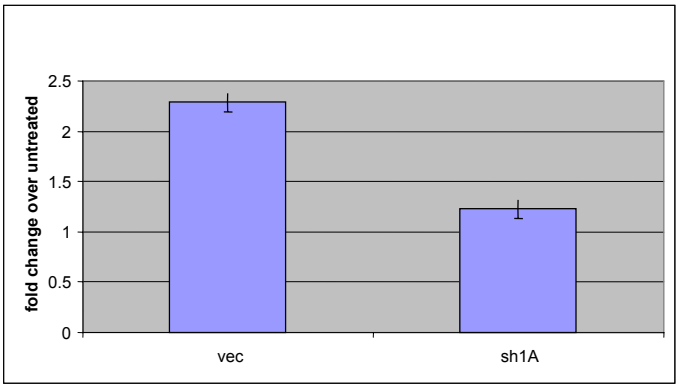
TASK2:

To use cell lines generated in TASK1 to determine the effects of RASSF1A levels on the action of Taxol on microtubules.

RASSF1A binds microtubules and promotes their stabilization/polymerization (4;5;8). Indeed, the effects on tubulin of adding RASSF1A to cells is reminiscent of the effects of treating them with Taxol (4). Moreover, down-regulation of RASSF1A makes cells more sensitive to Nocodazole, a microtubule destabilizing drug (5). Thus, we hypothesized that the presence of RASSF1A may be important to the ability of Taxol to induce microtubule polymerization. This would confirm RASSF1A loss as a component of the development of Taxol resistance in ovarian cancer and explain the results obtained in Figure 1.

When Taxol polymerizes, it becomes acetylated and this has been used as a marker for polymerization (9). The UC107 RASSF1A +/- matched pair of cell lines generated in TASK1 were treated with Taxol. After 48 hours the cells were lysed and equal quantities of protein subjected to Western analysis first for total tubulin and then for acetylated tubulin using an acetylated tubulin specific antibody. The ratio of acetylated tubulin to total tubulin was determined by densitometry scanning of the western blots to permit quantitative assessment of the effects of the presence of RASSF1A. Figure 5 shows that loss of RASSF1A expression reduces the ability of Taxol to promote microtubule polymerization (Figure 5).

Figure 5. The ability of Taxol to promote tubulin acetylation is dependent on RASSF1A.
UCI-107 RASSF1A +/- matched pair cells were treated with Taxol and after 48 hours lysed for analysis. Total protein levels in the lysates were measured and equalized and then the samples were subjected to Western analysis for total tubulin as well as acetylated tubulin using specific antibodies. The bands were quantified and average data expressed as a ratio of acetylated tubulin to total tubulin to give a fold change value. Knockdown of RASSF1A resulted in an approximately 50% reduction in the relative acetylation of tubulin. P = 0.042275 statistically significant (student's T-test, 2-tailed, paired)



Summary:

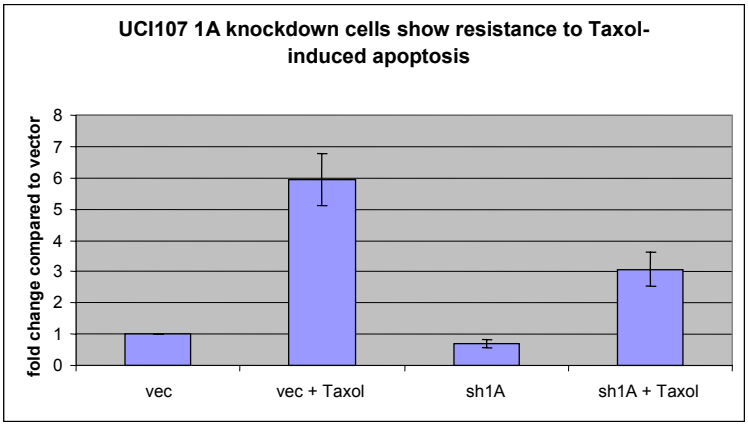
RASSF1A knockdown reduced the degree of acetylated tubulin produced by Taxol treatment in UC107 cells. This supports a role for the presence of RASSF1A as a necessary component for effective functioning of Taxol.

TASK3:

Using the cell systems established in TASK1, determine the effects of the RASSF1A status on the apoptotic response to Taxol.

RASSF1A is a pro-apoptotic protein and loss of RASSF1A expression may induce resistance to apoptosis (3;10). To determine if that may be the case in Ovarian cancer cells treated with Taxol, we took the RASSF1A +/- UCI-107 cells generated in the first task and treated them with Taxol. After 48 hours, the cells were assayed for apoptosis using the Promega Caspase 3/7 kit. This kit is a fluorescent assay for caspase activation, a hallmark of apoptosis. Figure 6 shows that knockdown of RASSF1A induces strong resistance to apoptosis induced by Taxol.

Figure 6. The role of RASSF1A in the response to Taxol, mediated apoptosis. The UCI-107 RASSF1A +/- matched pair of cells were treated with Taxol or carrier and after 48 hours lysed and assayed in a luminometer for caspase activation using a Promega Caspase 3/7 kit. Caspase activation, as measured by arbitrary luciferase units, in the RASSF1A knockdown cells was approximately half that of the RASSF1A wild type cells. Data presented is the average of two assays performed in triplicate. UCI-107 cells were treated for 22 hours with 50 nM Taxol before assay.
P = .0428 comparing vec + Taxol to sh1A + Taxol (student's T test, 2-tailed distribution)



Summary:

Down-regulation of RASSF1A in UCI-107 cells renders the cells resistant to Taxol induced apoptosis as well as well as Taxol induced microtubule polymerization.

TASK 4.

Testing the ability of two novel DNA Methyl Transferase (DNMT) inhibitors to synergize to restore the expression of epigenetically inactivated RASSF1A.

Treatment of several RASSF1A negative ovarian tumor cell lines showed that only the A547 cell line exhibited a detectable increase in the levels of RASSF1A protein when treated with the general DNMT (DNA Methyl Transferase Inhibitor) inhibitor 5-aza cytidine (5AzaC) (Figure 7).

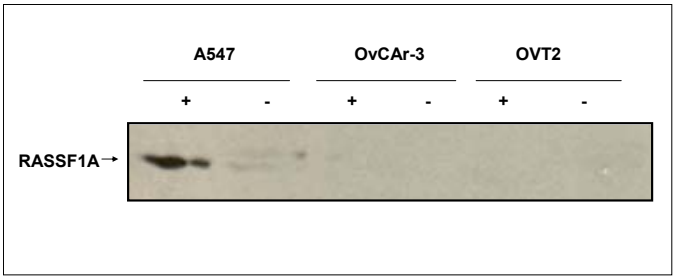


Figure 7. **The effects of 5AzaC on RASSF1A expression in ovarian tumor cell lines.** Three ovarian tumor cell lines that have been reported to be negative for RASSF1A expression were treated with the DNMT inhibitor 5AzaC for 72 hours. Cells were lysed, and the

lysates assayed for protein concentration. Equal quantities of protein were then subjected to Western analysis with a RASSF1A antibody. (+) = treated with 5AzaC, (-) = treated with carrier. Only the A547 cells exhibited a detectable increase in RASSF1A protein levels when treated with 5AzaC.

Thus, the A547 cells were selected for further study. RG108 is a novel DNMT inhibitor that was designed to specifically inhibit the enzyme DNMT1 (11). Zebularine is another novel DNMT inhibitor that has been shown to be active in restoring RASSF1A expression but is more specific and hence less toxic than 5-AzaC (12;13). Examination of the toxicity of RG108 and Zebularine allowed the determination of the minimal dose that provoked no detectable changes in cell growth or morphology. Combination of these two doses also resulted in no overt cell death (Figure 8).

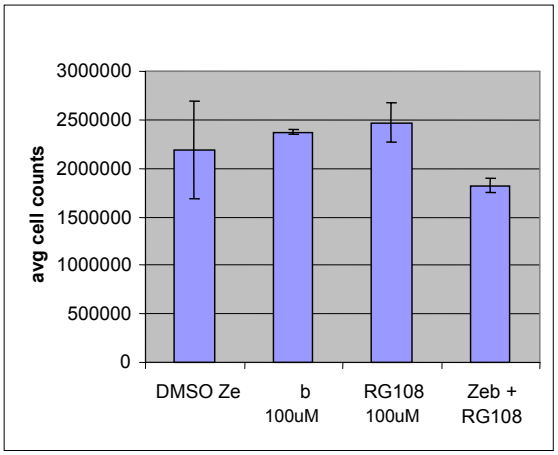


Figure 8. RG108 and Zebularine exhibit little or no toxicity either alone or in combination. Cells were treated with DimethylSulphoxide (DMSO) carrier, Zebularine (p=0.661), RG108 (p=0.544) or both (p=0.423) and counted after 48 hours. 2-tailed type 2 Students T test returns values showing no significant statistical difference compared to the DMSO treated cells.

Examination of the effects of the drugs on RASSF1A expression showed that RG108 and Zebularine could synergize to restore RASSF1A expression (Figure 9). This is the first demonstration of proof of principal for the use of combinations of different DNMT inhibitors with different modes of action in an epigenetic therapy regime

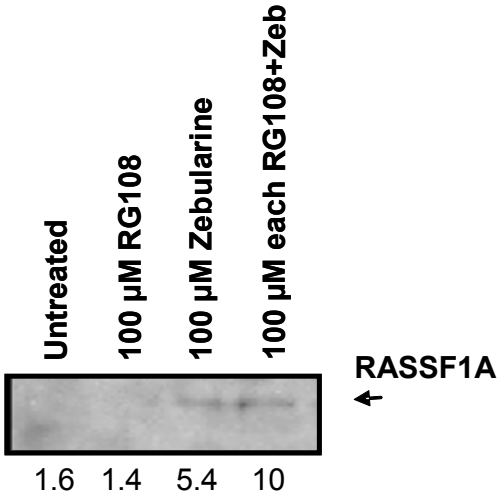


Figure 9. Synergistic reactivation of RASSF1A expression by RG108/Zebularine combinations.
A547 cells were treated with RG108, Zebularine or a combination of the two drugs. After 48 hours, the cells were lysed equal quantities of protein were immunoprecipitated with an anti-RASSF1A antibody. The IP was the subjected to Western analysis for RASSF1A. Densitometric quantification of the bands is shown below the Figure. The RG108 and Zebularine can synergize to restore RASSF1A expression to the cells.

Summary:

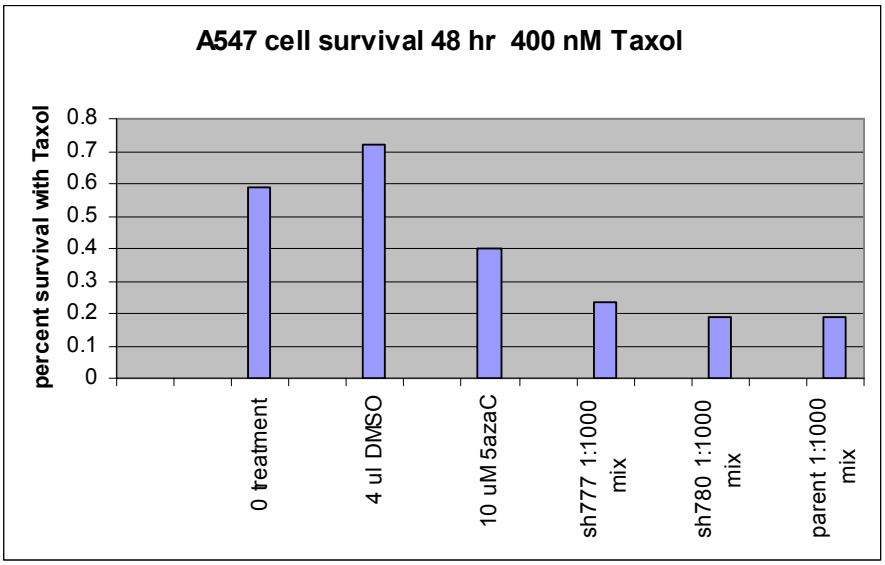
The two novel DNMT inhibitors RG108 and Zebularine operate using different mechanisms and can synergize to reverse the epigenetic inactivation of RASSF1A.

TASK 5

Use of the data obtained in TASK 4 to develop a synergistic epigenetic therapy regime to restore the sensitivity of RASSF1A negative Ovarian tumor cells to Taxol.

In task 4 we determined that RG108 and Zebularine could act synergistically to restore RASSF1A expression at doses that were too low to induce cell toxicity. Here, we treated the cells with the synergistic dose of RG108 and Zebularine and then added Taxol to the system. Figure 10 shows that we achieved enhanced tumor cell death with Taxol after pre-treatment with combined epigenetic therapy. Thus, we have achieved proof of principal that synergistic epigenetic therapy can complement the effects of Taxol on Ovarian cancer cells. Curiously, although this level of treatment can restore RASSF1A expression, we found that cells transfected with two different shRNAs against RASSF1A still exhibit synergistic cell death. Thus, the effects of the epigenetic therapy are probably being manifested by the activation of a panel of unknown genes, not just RASSF1A.

Figure 10. Synergistic epigenetic therapy enhances the Taxol response of ovarian tumor cells.
A547 cells transfected either with vector or shRNA to RASSF1A were treated with carrier (DMSO) or a combination of RG108 and Zebularine. The non-specific, relatively toxic DNMT inhibitor 5-Aza-C was included as a control. After 48 hours, Taxol was added at 400nM and the cells incubated for a further 48 hours. The cells were than stained with trypan blue to allow live cell quantification.



Summary:

Pre-treatment of A547 ovarian tumor cells with a synergistic, non-toxic combination of the novel DNMT inhibitors Zebularine and RG108 at a dose sufficient to reactivate RASSF1A sensitized the cells to the killing effects of Taxol. However, this effect did not appear to be dependent upon RASSF1A and so other tumor suppressor systems may also be invoked.

KEY RESEARCH ACCOMPLISHMENTS:

1. Determination that loss of RASSF1A expression renders some ovarian cancer cells resistant to Taxol and but not Cisplatin mediated cell death.

2. Determination that the full effects of Taxol on tubulin polymerization are dependent upon the presence of RASSF1A.

3. Determination that the effects of Taxol upon apoptosis induction are partially dependent upon the presence of RASSF1A.

4. Determination that different DNMT inhibitors with different mechanisms of action can synergize to restore expression of the RASSF1A protein.

5. Determination that epigenetic therapy can enhance the effects of Taxol on Ovarian cancer cells.

REPORTABLE OUTCOMES

None at this time

Conclusion

The RASSF1A tumor suppressor is frequently inactivated by an epigenetic process of aberrant promoter methylation in Ovarian cancer (1). RASSF1A complexes with microtubules and enhances their polymerization. Inactivation of RASSF1A results in an increased sensitivity to microtubule destabilizing drugs. Overall, the data suggests that RASSF1A plays an important role in the stabilization of microtubules. As the drug Taxol is thought to work in large part by stabilizing microtubules, and RASSF1A is frequently lost in ovarian tumors that become resistant to Taxol, we hypothesized that loss of RASSF1A might promote Taxol resistance. If so, we might be able to enhance Taxol sensitivity by restoring the expression of RASSF1A with drugs that block the aberrant methylation of its promoter. The results of the project support both these hypotheses. Moreover, we have found that the DNMT inhibiting drugs Zebularine and RG108 can synergize to reactivate RASSF1A, suggesting that it may be easier to obtain an effect with low, non-toxic doses of these drugs when administered together.

"So what": The results established proof of principal for the use of this novel combined epigenetic therapy with Taxol in Taxol resistant Ovarian tumors. Curiously, the data suggests that the effects of the DNMT inhibitors on the Taxol response may be independent of the RASSF1A status. This indicates that other, unknown genes modulated by the drugs are playing a role.

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APPENDIX

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Abstracts: None

Personnel:

Geoffrey J. Clark, Associate Professor

Susannah Kassler, Technician